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Human Natural Killer cell expression of ULBP2 is associated with a mature functional phenotype

Kiva Brennan [a], Brian P. McSharry [a,1], Sinéad Keating [a], Andreea Petrasca [b], Vincent P. O'Reilly [b], Joseph Keane [c], Derek G. Doherty [b], Clair M. Gardiner [a,*]

[a] NK Cell Laboratory, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, 152-160 Pearse Street, Trinity College, Dublin 2, Ireland
[b] Department of Immunology, School of Medicine, Trinity College Dublin, Dublin 8, Ireland
[c] Department of Clinical Medicine, School of Medicine, Trinity College Dublin, Dublin 8, Ireland

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ABSTRACT

NKG2D is an important activating receptor expressed on NK cells. Ligands (termed NKG2DL) for this receptor include ULBP1-6, MICA and MICB in humans; they are upregulated in stressed, cancerous or infected cells where they engage NKG2D to induce NK cell cytotoxicity and cytokine production.

Expression of NKG2DL on effector cells has been described in mice and more recently in human cells. We confirm that NK cell lines and IL-2 stimulated primary human NK cells also express the NKG2DL, ULBP2. However, expression of ULBP2 was not a result of transfer from a non-NK cell to an NK cell and in contrast to recent reports we saw no evidence that ULBP2 expression targeted these NK cells for fratricide or for cytotoxicity by NKG2D-expressing, non-NK effector cells.

ULBP2 expression was however linked to expression of mature $CD57^+$ NK cells. In particular, expression of ULBP2 was strongest on those NK cells that had evidence of recent activation and proliferation. We suggest that ULBP2 could be used to identify recently activated "mature" NK cells. Defining this phenotype would be useful for understanding the ontogeny on human NK cells.

1. Introduction

Natural Killer (NK) cells are important effector cells of the immune system which kill virally infected and transformed cells. NK cells express a range of activating and inhibitory receptors in order to interact with target cells and it is the net balance of signals through these receptors that determines NK cell function. A number of activating receptors on NK cells have been defined, the best described of which is NKG2D. NKG2D, also expressed on NKT cells, γδ T cells and activated $CD8^+$ T cells, is constitutively expressed on the surface of NK cells [1]. The current paradigm for regulating NK cell activation is based on expression of NKG2D ligands (NKG2DL) on the surface of stressed, cancerous or infected cells. These ligands engage NKG2D, resulting in activation of NK cell functions, including degranulation and cytokine production [2]. This mechanism of NK cell activation is critical in recognition and clearance of both cancer and virally-infected cells [3–6]. There have been many reports that cancer cells can secrete soluble NKG2DL to down-regulate NKG2D as an immune evasion mechanism [7,8] while recently a murine model has shown the opposite effect. Soluble NKG2DL, MULT1, stimulated NK cell activity and promoted tumour rejection [9]. This recent report highlights the importance of attaining a better understanding of the function of these proteins and their role in cancer clearance.

Two families of human NKG2DL exist: MHC-class I related proteins (MICA and MICB) and UL16-binding proteins (ULBP1-6). It is not known why there is such diversity of NKG2DL but it has been suggested that they are differentially expressed in various tissues in response to particular stresses. Regulation of NKG2DL expression on target cells is tightly controlled in order to maintain the balance of signals for NK cell activation and inhibition (reviewed in [10]).

Work in our laboratory revealed that human NK cells can be induced to express NKG2DL. We hypothesised that expression of NKG2DL on NK cells may provide a novel mechanism for regulation of NK cell functions. A recent paper has independently described NKG2DL expression on NK cells as a result of active transfer, termed trogocytosis, from target cells to the NK cell membrane

* Corresponding author.
E-mail address: clair.gardiner@tcd.ie (C.M. Gardiner).
[1] Current address: Discipline of Infectious Diseases and Immunology, Charles Perkins Centre, University of Sydney, NSW 2050, Australia.

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[11]. Furthermore, they demonstrate that expression of NKG2DL on NK cells targets them for cytotoxicity by NKG2D-expressing NK cells. Given that our data also demonstrates the expression of NKG2DL on human NK cells, but in response to activation rather than trogocytosis, we undertook to investigate the consequence of this activation-induced expression of NKG2DL on NK cell function.

2. Materials & methods

2.1. Cell Culture

The NK cell lines, NKL, YT and NK92 were maintained as previously described [12]. Daudi and 721.221 cells (ECACC) were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS. All media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies).

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of healthy volunteers from whom written and informed consent had been obtained and ethics approval granted by St. James's Hospital Ethics Committee. NKT cells and Vγ9Vδ2 T cells were expanded and cultured as previously described [13,14]. For cultured $CD8^+$ T cells, PBMCs (1×10^7 cells/ml) were cultured with immobilised 1 μg/ml anti-CD3 for 36–48 h, washed & cultured at a concentration of 5×10^5 cells/ml with 360 U/ml IL-2 for 5–7 days before use in experiments.

Highly purified NK cells were obtained using NK isolation kit II (Miltenyi Biotec), according to the manufacturer's instructions. Purity was assessed by flow cytometry with NK cells routinely purified to >90%. For NK cell sorting experiments, PBMCs were labelled with anti-CD56 and anti-CD3 and anti-ULBP2. $CD56^+$, $CD3^-$ cells were sorted on a BD FACSAria cell sorter from the lymphocyte gate, followed by $ULBP2^+$ and $ULBP2^-$ cell gating, purity was routinely 99.7–99.9%. Primary NK cells were cultured in RPMI supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37 °C in 5% CO_2.

2.2. Flow cytometry

Fluorescently conjugated antibodies against human proteins were purchased from BD Biosciences: anti-CD56 (B159), anti-CD3 (SK7), anti-CD69 (FN50), anti-NKG2D (1D11), anti-Ki67 (B56), anti-CD57 (HNK-1), anti-CD107a (H4A3), anti-IFNγ (25723.11). Flow cytometry was carried out on a Dako Cyan flow cytometer and analysed using FlowJo software (TreeStar).

2.3. NKG2DL staining

For unconjugated antibody staining, cells were incubated with unconjugated ULBP1 (AUMO1), ULBP2 (BUMO1), ULBP3 (CUMO3), MICA (AMO1), MICB (BMO1), MICA/B (BAMO1) antibody, or the corresponding isotype control Abs (all from BamoMab). Cells were subsequently incubated with 1/1000 goat anti-mouse AF488 or AF647 (Alexa). Non-specific binding of secondary antibody was blocked using BD Fc Block and cells were stained for CD56, CD3 and CD69. For NKG2D blocking experiments, anti-ULBP2 antibody was conjugated using DyLight 488 microscale labelling kit (Pierce/MSC).

2.4. Blocking experiments

Blocking of ULBP2 was carried out using a ULBP2 blocking antibody, AF1298, from R&D systems at a final concentration of 5 mg/ml for 30 min. Blocking of NKG2D was carried out as previously described [15,16]. Cells were incubated with 30 μg/ml anti-NKG2D (1D11, BD biosciences) for 30 min, cells were washed once prior to further treatment.

2.5. CD107a assay

PBMCs were stimulated, as required, in 96well-round-bottomed plates. Four h prior to harvesting, 721.221 cells (10:1 E:T ratio) and anti-CD107a-FITC were added to PBMCs and centrifuged at 150*g* for 5 min and incubated for 1 h. Ten μM monensin was added for a further 3 h before extracellular staining. For co-incubation experiments, 1×10^5 purified NK cells were incubated with a 1:1 ratio of expanded, purified iNKT, $CD8^+$ or γδ T cells.

2.6. IFNγ intracellular staining

Cells were stimulated as required, the last 4 h in the presence of Golgi-Plug (BD Pharmingen). Cell surface staining was performed, followed by intracellular staining with IFN-γ-FITC using the Cytofix/Cytoperm Plus kit (BD Pharmingen) according to the manufacturer's instructions.

2.7. NKG2D crosslinking

Flat-bottomed 96 well plates were coated with 5 μg/ml anti-NKG2D Ab (BD) overnight at 4 °C. A total of 2.5×10^5 sorted NK cells were added to each well with 1 μl/well GolgiStop, centrifuged at 300*g* and incubated at 37 °C for 3 h. Following incubation, cells were stained for CD107a, CD56, CD3 and intracellular IFNγ.

2.8. CFSE, Annexin V & 7-AAD staining

MACS-enriched NK cells were stimulated with IL-2 (500 U/ml) or left untreated for 2 days. Following stimulation, IL-2 treated NK cells were labelled with 0.03 μM CFSE (Invitrogen) for 10 min at room temperature, washed and incubated at a 1:1 ratio with unlabelled, unstimulated MACS-enriched NK cells for 6 h. Following co-incubation, NK cells were stained with Annexin V-APC (eBiosciences) and 7-AAD (Biolegend) as per manufacturer's instructions.

3. Results

3.1. Human NK cells can express "danger signals"

An unexpected observation of ULBP2 expression on NK cells during experiments in our laboratory prompted us to investigate the possibility that NK cell lines and primary NK cells expressed, or could be induced to express, ULBP2 and other NKG2DL. Analysis confirmed that NKL cells expressed ULBP2, ULBP1 and MICA. YT cells expressed ULBP2 but no other NKG2DLs, while NK92 cells did not express any of the NKG2DL tested (Fig. 1A middle and lower panels). ULBP3 was not expressed on any of the cell lines tested (Fig. 1A right-hand panels).

Following analysis of freshly-isolated, primary human NK cells, we observed no expression of any NKG2DLs (Fig. 1B, upper panels). However, there was a significant increase in the frequency of NK cells expressing ULBP2 in response to IL-2 stimulation compared with unstimulated cells (Fig. 1B, lower panels). Individual donor responses to IL-2 were varied, but NK cells from all donors consistently expressed ULBP2 following IL-2 stimulation (29.8% ± 2.6 SEM, n = 24) compared to unstimulated cells (5.5% ± 0.7) (Fig. 1C). While IL-12 and TNFα induced ULBP2 expression, these effects were much more modest and did not reach significance (Fig. 1D). ULBP2 expression on NK cells was not generally associated with NK cell activation as no increase was observed in response to